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(54) Title: ANTI-HYPERTENSIVE PEPTIDES

(57) Abstract: Provided are novel oligopeptides, two to four amino acid residues in length, derived from food protein digest products or chemically synthesized, which exhibit antihypertensive activity. These antihypertensive oligopeptides may be used as active ingredients in pharmaceutical compounds, dietary supplements and food ingredients. The present invention also relates to methods of treatment and prophylaxis of diseases such as myocardial infarction, left ventricularsystolic dysfunction, diabetes mellitus, progressive renal failure and congestive heart failure caused by or associated with hypertension using such novel antihypertensive oligopeptides.

ANTI-HYPERTENSIVE PEPTIDES

This application claims priority to copending United States provisional patent application Ser. No. 60/188,499, filed March 10, 2000, incorporated herein by reference.

Field of Invention

This invention relates to short oligopeptides, two to four amino acid residues in length, derived from food protein digest products or chemically synthesized, which exhibit antihypertensive activity. These novel antihypertensive oligopeptides may be used as active ingredients in pharmaceutical compounds, dietary supplements and food ingredients. The present invention also relates to methods of treatment and prophylaxis of hypertension and disease states such as myocardial infarction, left ventricular systolic dysfunction, diabetes mellitus, progressive renal failure and congestive heart failure caused by or associated with hypertension using such novel antihypertensive oligopeptides.

Background of the Invention

Hypertension, generally defined as abnormally increased blood pressure, is a disease which is estimated to affect approximately 50 million people in the United States and 170 million people worldwide. It is clinically recognized as an elevation of systolic arterial blood pressure of 140 mm Hg or greater and an elevation of diastolic arterial blood pressure of 90 mm Hg or higher.

Hypertension may be caused by a number of different physiological mechanisms and no single or specific cause is known for the hypertension referred to as primary (essential) hypertension. Primary hypertension has been attributed to such causes as hemodynamic pattern, genetic predisposition, vascular hypertrophy, hyperinsulinemia, defects in cell transport of binding, defects in the renin-angiotensin system (low-renin or high-renin hypertension) and along with insulin, angiotensin and natriuretic hormone, catecholamines arising in response to stress are known to be pressor-growth promoters. Increased sympathetic nervous activity may also raise the blood pressure in a number of ways, either alone or in concert with stimulation of renin release by catecholamines, thus causing arteriolar and venous constriction by increasing cardiac output or by altering the normal renal pressure-volume relationship. Also, recent evidence demonstrates that sodium has a causal role in the genesis of hypertension.

Primary hypertension is also associated with various disease states for example, obesity, sleep apnea, physical inactivity, alcohol intake, smoking, diabetes mellitus, polycythemia and gout. Secondary forms of hypertension may arise from oral

contraceptive use and parenchymal renal disease, for example, renovascular hypertension may be caused by atherosclerotic disease, tumors (renin-secretory tumors), Cushing's Syndrome, heart surgery and pregnancy. Chronic hypertension and renal disease during pregnancy may progress into eclampsia which is a primary cause of fetal death. Furthermore, primary hypertension is a relatively common disease state in humans and has been associated with the early onset of coronary disease, kidney failure and stroke. Primary hypertension is generally asymptomatic and has been termed a silent killer.

One regulator of hypertension, the renin-angiotensin mechanism, has been extensively studied and its effects on hypertension are well documented. In this mechanism, angiotensinogen is secreted by the liver and is cleaved by renin to yield the biologically inactive angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) (SEQ ID NO: 1). The last two residues of angiotensin I are removed by the hydrolytic action of angiotensin converting enzyme (ACE) to form angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) (SEQ ID NO: 2) which exhibits a strong vasopressor action. Specifically, angiotensin II increases blood pressure by contracting the smooth muscles of the blood vessel walls and promoting secretion of aldosterone by action on the adrenal cortex. In addition, angiotensin II further acts to increase blood pressure by decomposing and inactivating bradykinin, a peptide which lowers blood pressure. Since ACE not only produces a peptide which increases blood pressure (angiotensin II) but also decomposes and inactivates a peptide which decreases blood pressure (bradykinin), inhibiting the activity of ACE is an effective way of depressing blood pressure.

Several pharmaceuticals have been studied for their ability to reduce blood pressure. Much focus has been directed on developing compounds which inhibit the function of ACE in the renin-angiotensisn mechanism. However, while the inhibition of ACE affects blood pressure, reduction in blood pressure often can not be explained by the inhibition of ACE alone. Thus antihypertensive compounds may reduce blood pressure through other mechanisms or through a combination of physiologic mechanisms.

Currently, hypertension is often treated with the use of anti-hypertensive pharmaceutical products including calcium channel blockers, beta blockers, diuretics, alpha blockers, central alpha agonists, angiotensin II antagonists and ACE inhibitors, such as Captopril and Enalapril. While such pharmaceutical products are effective in decreasing elevated blood pressure, they often have negative side effects associated with their use. Some common side effects include increased cholesterol and glucose levels associated with diuretics, sedation and dry mouth associated with central alpha agonists, bronchospasm and bradycardia associated with beta blockers, and conduction defects associated with calcium agonists. Furthermore, sudden blood pressure elevation and/or

other unsafe side effects may occur after one ceases taking such antihypertensive pharmaceuticals.

Alternatively, several compounds produced from food stuffs have been studied for their ability to reduce blood pressure. These ACE inhibitory substances from natural sources such as milk protein, soybean protein or fish meat protein are proposed for practical use as antihypertensive agents having low toxicity and great safety. Proteins from natural resources are important for supplying nutrients and energy and these functions have been defined as a primary function of protein. However, many studies have shown that proteins perform other functions relating to physiological regulation. For example, peptides from natural sources provide other beneficial effects such as promoting calcium absorption and regulating serum cholesterol.

Thus, it would be beneficial to treat hypertension with antihypertensives obtained from natural sources. However, most of these anti-hypertensive substances are contained only in small amounts in such natural products and therefore sufficient effect cannot be expected in the practical oral intake of such natural products. A further problem is that many naturally obtained peptides do not have strong anti-hypertensive effects even if the peptides have strong ACE inhibition activity.

Currently, there are few non-pharmaceutical products for treating hypertension in the nutritional or dietary supplement markets. Several studies, however, have disclosed particular oligopeptides produced from a variety of protein sources which exhibit antihypertensive activity. For example, ACE inhibiting substances have been isolated from microorganisms and various foods and studied for their potential as hypotensive agents (Kunio Suetsuna, "Hakko to Kogyo" (Fermentation and Industry) 46 (No. 3), 179-182 (1988). Furthermore, a number of functional peptides derived from milk, soy, corn and fish protein have been identified. *See e.g.,* U.S. Patent Nos. 5,238,932 and 5,071,955; Maruyama, S., Biosciences and Industry, 47:38-42 (1989); Maruyama et al., Lecture Gists for the 1988 Year Great Annual Meeting of Nippon Hakko Kogaku Kai (Japan Fermentation Engineering Society), p. 23 (1988); Maruyama et al., Lecture Gists for the 1989 Year Meeting of Nippon Nogei Kagaku Kai (Japan Society for Bioscience, Biotechnology and Agrochemistry), p. 8 (1989); Miyoshi et al., Gists for the 1989 Year Meeting of Nippon Eiyo Shokuryo Gakki (Japan Nutrition and Food Society) p. 113 (1989); and Miyoshi et al., Nippon Nogei Kagaku Kaishi, Journal of Japan Agricultural Chemistry Society 64:555 (1990) (Lecture Gists for the 1990 Year Great Annual Meeting); Hiroyuki Ukeda, Nippon Nogei Kagaku Kaishi, Journal of Japan Society for Bioscience, Biotechnology, and Agrochemistry, 66:25-29 (1992). Moreover, anti-hypertensive oligopeptides have been isolated from sardine and tuna muscle, dairy products, corn protein, soybean products, yeast, and other plant and animal proteins. *See*

Astawan, E. et al., Biosci. Biotech. Biochem., 59:425-429 (1995); Ariyoshi, A., "Angiotensin-Converting Enzyme Inhibitors Derived from Food Proteins, Trends in Food," Science & Technology 4:139-144, May 1993; and Yokoyama, K. et al., Biosci. Biotech. Biochem., 56:1541-1545 (1992).

Recently, two tripeptides reported as having strong ACE inhibiting activity, Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) have been derived from lactic acid bacteria-fermented milk. *See* Nakamura et al., J. Dairy Sci. 78: 777-783 (1995). Furthermore, these tripeptides have been reported to exhibit strong antihypertensive effect in spontaneously hypertensive rats (SHR). *See* Nakamura et al, J. Dairy Sci., 78: 1253-1257 (1995). However, since the tripeptides are produced by proteinase which is produced by lactic acid bacteria as lactic acid fermentation proceeds in milk, the resulting amount of tripeptides tends to vary depending on the conditions of fermentation. It is thus difficult to obtain a consistent amount of the tripeptides.

Another antihypertensive peptide, Leu-Lys-Pro-Asn-Met (LKPNM) (SEQ ID NO: 3), has been isolated from dried bonito digested in thermolysin. Dried bonito is a traditional Japanese seasoning made of skipjack tuna (bonito) muscle. In the manufacturing process of dried bonito, bonito meat is treated with fungi. Thermolysin digests of non-dried bonito protein does not produce the LKPNM oligopeptide that is found in the thermolysin digest of dried bonito. *See* Yokoyama et al., Biosci. Biotech. Biochem. 56: 1541-1545 (1992). After LKPNM enters the body, it is believed to be further converted into the peptide LKP and NM in the digestive organs or blood. The LKP peptide fragment exhibits the greatest anti-hypertensive properties and is therefore believed to be a principal active form of LKPNM. LKP has been isolated from digestion of fish muscle, corn protein, soybeans, and milk products.

Other peptides obtained from natural sources may also demonstrate anti-hypertensive activities. For instance, LKPN (SEQ ID NO: 4) is another peptide subunit that is an enzymatic product of LKPNM and also exhibits anti-hypertensive properties. LKPN has been isolated from the enzymatic digestion of fish muscle and soybeans. *See* Suetsuna et al., Kiso to Rinsho (Clinical Report) 25: 477304784 (1991); Japanese Application No. 7138287. However, most of these antihypertensive peptides are contained only in small amounts in such natural products and therefore, a sufficient effect on hypertension cannot be expected in practical oral intake.

Thus it would be desirable to identify natural antihypertensive peptides which are effective in oral dosage and have low toxicity and great safety without the negative side effects associated with current antihypertensive pharmaceutical products. Such peptides can be employed as the active ingredient in dietary supplements and food ingredients in order to reduce either systolic or diastolic blood pressure levels.

Summary of Invention

Accordingly, an object of the present invention is to provide compositions comprising a peptide selected from the group consisting of Lys-Pro-Asn-Met (KPNM) (SEQ ID NO: 5), Lys-Pro-Asn (KPN), Pro-Asn-Met (PNM), Leu-Lys (LK), Pro-Asn (PN) and Asn-Met (NM) or their acceptable acid or base addition salt, together with a pharmaceutically suitable diluent.

Another object of the present invention is to provide edible compositions comprising a foodstuff comprising a peptide selected from the group consisting of KPNM, KPN, PNM, LK, PN and NM and their acceptable acid or base addition salts.

It is yet another object of the present invention to provide edible compositions comprising a nutritional substance and an oligopeptide selected from the group consisting of KPNM, KPN, PNM, LK, PN and NM or their acceptable acid or base addition salt.

Yet a further object of the present invention is to provide methods of reducing various diseases such as myocardial infarction, left ventricular systolic dysfunction, diabetes mellitus, progressive renal failure and congestive heart failure caused by or associated with hypertension which comprises administering an antihypertensively effective amount of an oligopeptide selected from the group consisting of KPNM, KPN, PNM, LK, PN and NM or their pharmaceutically acceptable acid or base addition salt.

Other features of the present invention will be in part apparent to those skilled in the art and in part pointed out in the detailed description provided below.

Brief Description of Drawings

Figure 1 is a graph representing the effect of a 10 μmol intravenous dose of LKPNM and its peptide fragments on systolic blood pressure in spontaneous hypertensive rats.

Figure 2 is a graph representing the effect of a 5 μmol intravenous dose of LKPNM and its peptide fragments on systolic blood pressure in spontaneous hypertensive rats.

Figure 3 is a graph representing the effect of a 10 μmol oral dose of LKPNM and its peptide fragments on systolic blood pressure in 27 week old spontaneous hypertensive rats.

Figure 4 is a graph representing the effect of a 10 μmol oral dose of LKPNM and its peptide fragments on systolic blood pressure in 13 week old spontaneous hypertensive rats.

Detailed Description of Invention

All publications, patents, patent applications or other references cited in this application are herein incorporated by reference in their entirety as if each individual publication, patent, patent application or reference are specifically and individually indicated to be incorporated by reference.

Abbreviations and Definitions

To facilitate understanding of the invention, a number of terms as used herein are defined below:

The amino acid residues are abbreviated herein according to convention to their single letters symbols and/or their three letter symbols: A and Ala represent alanine; R and Arg represent arginine; N and Asn represent asparagine; D and Asp represent aspartic acid; C and Cys represent cysteine; Q and Gln represent glutamine; E and Glu represent glutamic acid; G and Gly represent glycine; H and His represent histidine; I and Ile represent isoleucine; L and Leu represent leucine; K and Lys represent lysine; M and Met represent methionine; F and Phe represent phenylalanine; P and Pro represent proline; S and Ser represent serine; T and Thr represent threonine; W and Trp represent tryptophan; Y and Tyr represent tyrosine; and V and Val represent valine.

As used herein, "ACE" means angiotensin converting enzyme.

As used herein, "edible composition" is defined as compositions which may be ingested by a mammal such as foodstuffs, nutritional substances and pharmaceutical compositions. As used herein "foodstuffs" refer to substances that can be used or prepared for use as food for a mammal and include substances that may be used in the preparation of food (such as frying oils) or food additives. For example, foodstuffs include animals used for human consumption or any product therefrom, such as, for example, eggs. Typical foodstuffs include but are not limited to beverages, (*e.g.*, soft drinks, carbonated beverages, ready to mix beverages), infused foods (*e.g.* fruits and vegetables), sauces, condiments, salad dressings, fruit juices, syrups, desserts (*e.g.*, puddings, gelatin, icings and fillings, baked goods and frozen desserts such as ice creams and sherbets), soft frozen products (*e.g.*, soft frozen creams, soft frozen ice creams and yogurts, soft frozen toppings such as dairy or non-dairy whipped toppings), oils and emulsified products (*e.g.*, shortening, margarine, mayonnaise, butter, cooking oil, and salad dressings) and intermediate moisture foods (*e.g.*, rice and dog foods).

The term "substantially pure" when referring to oligopeptides, denotes those oligopeptides that are largely or wholly separated from the proteins or other contaminants with which they are naturally associated. An oligopeptide is considered substantially pure when that oligopeptide makes up greater than about 50% of the total content of the

composition containing the oligopeptide and typically, greater than about 60% of the total content of the composition containing the oligopeptide. More typically, a substantially pure oligopeptide will make up from about 75% to about 90% of the total composition containing the oligopeptide. Preferably, the oligopeptide will make up greater than about 90%, and more preferably, greater than about 95% of the total composition containing the oligopeptide.

As used herein, the terms "anti-hypertensively effective amount" shall mean an amount sufficient to reduce, inhibit, or prevent hypertension in a subject.

As used herein, the terms "treatment" or "treating" relate to any treatment of hypertensive disease and include: (1) preventing hypertension from occurring in a subject who may be predisposed to the disease but who has not yet been diagnosed as having it; (2) inhibiting the disease, *i.e.*, arresting its development; or (3) ameliorating or relieving the symptoms of the disease, *i.e.*, causing regression of the hypertensive state.

Accordingly, the present invention is directed to a set of short oligopeptides Lys-Pro-Asn-Met (KPNM), Lys-Pro-Asn (KPN), Pro-Asn-Met (PNM), Leu-Lys (LK), Pro-Asn (PN) and Asn-Met (NM) which exhibit antihypertensive activity and effectively reduce blood pressure. Unlike many current antihypertensive pharmaceutical compounds, these naturally-derived antihypertensive oligopeptides do not exhibit the adverse side effects or alter normal blood pressure. These oligopeptides may be either derived from food protein digest products or chemically synthesized.

The present invention also relates to compositions comprising an antihypertensively effective amount of any of the oligopeptides KPNM, KPN, PNM, LK, PN and NM or their pharmaceutically acceptable acid or base addition salt. Examples of the pharmaceutically acceptable acid addition salts include but are not limited to salts formed with an inorganic acid such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and salts formed with an organic acid such as acetic acid, propionic acid, glycolic acid, lactic acid, malic acid, tartaric acid, citric acid, ascorbic acid, maleic acid, oxalic acid, fumaric acid, succinic acid, malonic acid, methanesulfonic acid, benzensulfonic acid, toluene-sulfonic acid.

These unique oligopeptides have demonstrated significant antihypertensive activity and therefore, are useful in the treatment and prophylaxis of various disease states such as left ventricular systolic dysfunction, myocardial infarction, diabetes mellitus and progressive renal impairment/failure caused by or associated with hypertension.

Preparation of Oligopeptides

The oligopeptides of the invention may be prepared by a process designed to hydrolyze or digest the selected protein with a cleaving agent such as a protease. First, a

source protein such as milk, soybeans, corn, wheat, rice, peanuts, beef muscle, lamb muscle, pork muscle, turkey muscle, chicken muscle, fish muscle other than dried bonito fish, yeast, bacteria, or products thereof is mixed with a suitable liquid such as sterilized water to form a homogeneous mixture. The pH of the homogeneous mixture is adjusted to a pH that is favorable for a selected cleaving agent. The pH can, if necessary, be adjusted during the course of the reaction with an aqueous sodium hydroxide solution, hydrochloric acid or the like. The homogeneous mixture is then boiled and the cleavage agent is added to digest the protein present in the mixture. In order to promote protein digestion, the temperature of the reaction mixture during the reaction is cooled and maintained within approximately 20°C to 50°C, preferably 30°C to 40°C, and most preferably about 37°C.

An endo type protease such as thermolysin, pepsin, trypsin, chymotrypsin, Proteinase E, Proteinase K and Actinase E are preferred for use to digest the protein, with thermolysin being particularly preferred. The optimal concentration of the cleaving agent within the digestion mixture is dependent upon the cleaving agent selected. Although the addition amount of the cleavage agent depends on its titer, the amount is usually 0.01% by weight or more, preferably 0.1 to 10% by weight based on the protein, preferably a final concentration of approximately 880 μg/ml based on the amount of protein. It is also possible to add part of the cleavage agent during the course of the reaction.

Treatment of the protein with the cleavable agent is usually carried out in water or in a suitable buffer (for example, a Tris-HCl buffer or a phosphate buffer). While the concentration of the protein substrate is not particularly limiting so long as it is possible to carry out stirring and mixing, it is preferable that the concentration of the protein substrate is in the range of 2 to 20% (w/v). The reaction time also varies depending on the cleavage agent used, addition amount of the enzyme, reaction temperature, reaction pH and the desired oligopeptide to be isolated but is preferably in the range of 1 to 5 hours.

Discontinuance of the hydrolysis reaction can be made according to a known method. Such methods which may be used to inactivate the hydrolysis reaction include but are not limited to inactivation of the cleavage agent either by heating of the hydrolyzate; altering the pH with the addition of an organic acid such as citric acid or malic acid, an inorganic acid such as hydrochloric acid or phosphoric acid or an alkali such as sodium hydroxide or potassium hydroxide; or the separation of the cleaving agent from the hydrolyzate by filtration using an ultrafiltration membrane.

Once the cleaving agent is inactivated, the digestion mixture is cooled and the resulting hydrolyzate solution is subjected to solid-liquid separation (for example, centrifugation or filtration) and the resulting liquid is fractionated by ultrafiltration, gel filtration or other commonly used methods in order to obtain a liquid portion containing a fraction having a molecular weight of 10,000 or less. This liquid or its concentrate (for

example, freeze-dried product) contains the oligopeptides of the invention and is further fractionated to obtain each desired oligopeptide.

The liquid containing the desired oligopeptide is further separated into solid and liquid phases by centrifugation. The supernatant is removed and may be transferred to another centrifuge tube to be centrifuged a second time. The solid phases obtained by centrifugation are dried and a mobile phase solvent is added to resuspend the dried contents in the centrifuge tubes in order to form a mobile phase mixture. The mobile phase mixture is then centrifuged and the resulting supernatant containing the desired oligopeptides is fractionated by chromatographic methods such as liquid chromatography, HPLC, or the like. The desired oligopeptides are then isolated according to their respective retention times.

The oligopeptides of the present invention can alternatively be prepared by other synthetic methods such as liquid-phase peptide synthesis or solid-phase peptide synthesis. Approximate peptide synthesis methods are described in Nobuo Izumiya, Tetsuo Kato, Haruhiko Aoyagi and Michinori Waki, "The Foundations and Experiments of Peptide Synthesis" (Pepuchido Gosei no Kiso to Jikken) published by Maruzen Co., Ltd., Tokyo, 1985, incorporated herein by reference. Methods for carrying out liquid phase synthesis of peptides coupled to a soluble oligomeric support are well known by those skilled in the art. *See e.g.*, Bayer, Ernst and Mutter, Manfred, Nature 237: 512-513 (1972); Bayer, Ernst, et al., J. Am. Chem. Soc. 96: 7333-7336 (1974); Bonora, Gian Maria, et al., Nucleic Acids Res. 18: 3155-3159 (1990).

Solid-phase peptide synthesis entails the sequential assembly of the appropriate amino acids into a peptide of a desired sequence while the end of the growing peptide is linked to an insoluble support. Usually, the carboxyl terminus of the peptide is linked to a polymer from which it can be liberated upon treatment with a cleavage reagent. In a common method, an amino acid is bound to a resin particle, and the peptide generated in a stepwise manner by successive additions of protected amino acids to produce a chain of amino acids. Modifications of the technique described by Merrifield are commonly used. *See, e.g.*, Merrifield, J. Am. Chem. Soc. *96*: 2989-93 (1964), incorporated herein by reference.

In an automated solid-phase method, peptides are synthesized by loading the carboxy-terminal amino acid onto an organic linker (*e.g.*, PAM, 4-oxymethyl phenylacetamidomethyl), which is covalently attached to an insoluble polystyrene resin cross-linked with divinyl benzene. The terminal amine may be protected by blocking with t-butyloxycarbonyl. Hydroxyl- and carboxyl-groups are commonly protected by blocking with O-benzyl groups. Synthesis is accomplished in a commercially available automated peptide synthesizer. *See e.g.*, Model 430-A, Applied Biosystems, Foster City, Calif.

Following synthesis, the product may be removed from the resin and the blocking groups are removed by using hydrofluoric acid or trifluoromethyl sulfonic acid according to established methods. *See e.g.*, Bergot and McCurdy, Applied Biosystems Bulletin (1987) incorporated herein by reference. A routine synthesis may produce 0.5 mmole of peptide-resin. Following cleavage and purification, a yield of approximately 60 to 70% is typically produced. Purification of the product peptides may be accomplished by crystallizing the peptide from an organic solvent such as methyl-butyl ether, dissolving in distilled water and using dialysis or reverse high-pressure liquid chromatography. Purified peptide may be lyophilized and stored in a dry state until use. Analysis of the resulting peptides may be done using the common methods of analytical high pressure liquid chromatography (HPLC) and electrospray mass spectrometry (ES-MS).

Pharmaceutical Compositions

The present oligopeptides and their acid or base addition salts have antihypertensive activity and thus are effective for treatment and prophylaxis of hypertension of mammals including human beings. Administration of the desired oligopeptide and/or its acid or base addition salts may be in the form of a pharmaceutical composition and at least one pharmaceutical auxiliary and shall be in an amount to effectively reduce hypertension in a subject. The present oligopeptides and their acid or base addition salts can be administered parenterally, namely by intravenous injection, rectal administration or orally, and formulated into a form suitable for each administration method.

Pharmaceutical compositions comprising any of the antihypertensive oligopeptides KPNM, KPN, PNM, LK, PN and NM or their pharmaceutically acceptable acid or base addition salts may be administered to a subject in order to effectively reduce hypertension. Examples of the pharmaceutically acceptable acid addition salts include but are not limited to salts formed with an inorganic acid such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and salts formed with an organic acid such as acetic acid, propionic acid, glycolic acid, lactic acid, malic acid, tartaric acid, citric acid, ascorbic acid, maleic acid, oxalic acid, fumaric acid, succinic acid, malonic acid, methanesulfonic acid, benzensulfonic acid, toluene-sulfonic acid.

The acid addition salts of the present oligopeptides can be prepared according to a conventional method. For example, an acid addition salt can be obtained by reacting one of the present oligopeptides containing a basic amino acid residue with a suitable acid in one equivalent amount thereto in water and then freeze-drying the product.

Furthermore, alkali or alkaline earth metal salts, ammonium salts or organic base salts (hereinafter these are referred to as base salts) can also be prepared according to a

conventional method. For example, a base salt can be obtained by reacting one of the present oligopeptides containing an acidic amino acid residue with a suitable base in one equivalent amount thereto in water and then freeze-drying the product.

The effective dosage of the desired oligopeptide will depend partly on the route of administration of the pharmaceutical composition. If the oral route is employed, the absorption of the composition will be an important factor. The effective amount of the desired oligopeptide or its pharmaceutically acceptable or base addition salt in the antihypertensive composition is approximately 1 to 100% (w/w), preferably 10 to 100% (w/w). Preferably, an antihypertensively effective amount of the desired oligopeptide or its pharmaceutically acceptable addition salt is approximately 10 to 200 mg/kg/day when administered to a mammal including humans beings for the purpose of treatment or prophylaxis of hypertension.

Formulations may be prepared in a manner suitable for systemic administration or local administration. Systemic formulations include those designed for injection (e.g., intramuscular, intravenous or subcutaneous injection) or may be prepared for transdermal, transmucosal, or oral administration. The formulation will generally include a pharmaceutically acceptable diluent as well as, in some cases, adjuvants, buffers, preservatives and the like.

Pharmaceutical forms for injections usually include sterilized aqueous solutions. Pharmaceutical formulations for injection can further contain pharmaceutical auxiliaries other than water such as a buffering and a pH adjusting agent (e.g., sodium hydrogenphosphate, citric acid), a tonicity agent (e.g., sodium chloride, glucose), a preservative (methyl p-hydroxybenzoate, propyl-hydroxybenzoate or the like). The pharmaceutical formulations can be sterilized by filtration through a bacteria-holding filter, incorporation of a sterilant into the composition, or irradiation or heating of the composition. The pharmaceutical formulations can also be prepared by first preparing and sterilizing a solid pharmaceutical composition and dissolving the solid composition at the time of use in sterilized water for use as an injection.

Orally administered agents are prepared in a form suitable for absorption in gastrointestinal organs. Tablets, capsules, granules, fine granules and powders can contain conventional pharmaceutical auxiliaries such as a binder (e.g., syrup, gum arabic, gelatin, sorbitol, tragacanth, polyvinylpyrrolidone or hydroxycellulose), an excipient (e.g., lactose, sucrose, corn starch, calcium stearate, sorbitol or glycine), a lubricant (e.g., magnesium stearate, talc, polyethylene glycol or silica), a disintegrant (potato starch or carboxymethylcellulose), or a wetting agent (e.g., sodium lauryl sulfate). Tablets can be coated using any conventional method. Oral liquid agents may also contain additives such as a preservative (methyl p-hydroxybenzoate, propyl p-hydroxybenzoate or sorbic acid).

Furthermore, edible compositions containing the antihypertensive oligopeptides and their acid or base addition salts can also be ingested as an additive or supplement contained in foods and drinks. These oligopeptides can be formulated together with a nutritional substance such as various vitamins and minerals and incorporated into substantially liquid compositions such as nutrient drinks, soymilks and soups; substantially solid compositions; and gelatins or used in the form of a powder to be incorporated into various foods. The content of the antihypertensive effective ingredient in such a functional or health food can be similar to the dose contained in a typical pharmaceutical agent.

The following examples illustrate the invention, but are not to be taken as limiting the various aspects of the invention so illustrated.

EXAMPLES

Example 1: Preparation of Antihypertensive OligoPeptides from Natural Bonito Fish

Dried bonito, Calpis milk, and soybeans were digested and the oligopeptide sequences of LKPNM and its enzymatically cleaved subunits were isolated and the peptide quantities compared according to the procedure outlined below.

Five grams of protein (dried bonito muscle, Calpis milk or soybeans) were added to 45 ml of distilled water and homogenized for a period of 1 to 2 minutes using a Polytron homogenizer (Model PT-3100, Kinematica AG, Littan, Switzerland). The homogenized mixture was adjusted to a pH of 7.0 using sodium hydroxide. The neutralized homogenized mixture was boiled for a period of 10 minutes. Thermolysin was added to the mixture to a final concentration of 880 μg/ml. The mixture was held at a temperature of 37°C and enzymatically digested for a period of 0, 1, 3, and 5 hours. The enzymatic reaction was stopped at the end of each digestion period by boiling the mixture for 10 minutes to inactivate the thermolysin. The homogenized mixture was transferred to centrifuge tubes and centrifuged at 3000 rpm at 4°C for a period of 15 minutes. 200 μl of the supernatant was collected and placed into a clean microfuge tube and centrifuged at 8000 rpm at 10-40°C for a period of 10 minutes. The contents of the microfuge tube were dried down under nitrogen gas (N_2) at a temperature of 37°C. The dried contents of the microfuge tube were then resuspended in 100 μl of the mobile phase solvent (water) and centrifuged at 8000 rpm at 10-40°C for a period of 10 minutes. The resulting supernatant was transferred to an HPLC vial and 25 μl of the supernatant was injected into the HPLC. The LKPNM and its oligopeptide fragments were isolated and quantified.

Example 2: Preparation of Oligopeptides From Protein Sources

Protein from pork muscle, beef muscle, chicken muscle, turkey muscle, fish muscle and soybeans were each digested in pepsin, chymotrypsin, trypsin, and thermolysin. From each digest, LK, LKPN, KPNM, PNM, LKP and LKPNM were quantified and identified in Tables 1-6.

Five grams of protein (pork muscle, beef muscle, chicken muscle, turkey muscle, fish muscle and soybeans) were added to 45 ml of distilled water and homogenized. The homogenized mixture was adjusted to pH 2.0 with hydrochloric acid for pepsin digestion. For chymotrypsin, trypsin, and thermolysin digestion, the homogenized mixture was adjusted to pH 7.0 using sodium hydroxide. The homogenized mixtures were boiled for a period of 10 minutes. A cleaving agent, pepsin, chymotrypsin, trypsin, and thermolysin, was then added to the mixtures in a quantity resulting in a final concentration of 800 μg/ml. The mixture was held at a temperature of 37°C and enzymatically digested for a period of 0, 1, 3, and 5 hours. The enzymatic reaction was stopped at the end of each digestion period by boiling the mixture for 10 minutes to inactivate the cleaving agent. The mixture was transferred to centrifuge tubes and centrifuged at 1500 rpm at a temperature of about 30°C for a period of 10 minutes. The supernatant was removed and analyzed using liquid chromatography/mass spectroscopy (LC/MS) to quantify the presence of oligopeptide fragments.

The supernatant was diluted 1:10 in deionized water with 0.1% trifluoroacetic acid (TFA)to form samples for LC/MS injections. 10 μg samples were injected into an LC/MS (Hewlett-Packard 1100 HPLC/MSD System, Palo Alto, CA) operated in the positive ion electrospray ionization (ESI) mode. Components of the samples were separated using a reverse phase HPLC method and a Waters C_{18} Symmetry column operated at 70°C. Positive ESI mass spectra of standard reference materials showed that the major ions formed were MH^+. This mass was monitored to quantify oligopeptides present in the samples. Water and acetonitrile contained 0.1% TFA. The water/acetonitrile flow rate was 1.0 mL/min with a gradient that began at 0% acetonitrile which increased linearly to 100% acetonitrile between 4 and 10 minutes. A 2 minute post time was used to allow the column to equilibrate between injections.

The oligopeptide concentrations were calculated using the following formula:

$$Concentration(\mu g / g) = \frac{ExtractConcentration(\mu g / mL) \times ExtractVolume(mL)}{SampleSize(g)}$$

Tables 1-6 summarize the quantities of the oligopeptides identified from the pepsin, chymotrypsin, trypsin, and thermolysin digestions of the protein.

Table 1: Oligopeptides Released from Enzyme Digested Pork

Oligopeptide	Pepsin	Chymotrypsin	Trypsin	Thermolysin
LKPNM	0.2			0.1
LKPN	1			0.7
LKP	0.3			0.1
LK	0.4	0.9	0.5	0.3
KPNM				
PNM	0.4			0.2

yield rate > 0.1 mg/g fresh food

Concentration of oligopeptides released = mg oligopeptide/g fresh food

Table 2: Oligopeptides Released from Enzyme Digested Beef

Oligopeptide	Pepsin	Chymotrypsin	Trypsin	Thermolysin
LKPNM	0.2			0.1
LKPN	1.2			0.9
LKP	0.4			0.2
LK	0.4	0.8	0.4	0.2
KPNM	0.2			
PNM	0.5	0.1	0.1	0.2

yield rate > 0.1 mg/g fresh food

Concentration of oligopeptides released = mg oligopeptide/g fresh food

Table 3: Oligopeptides Released from Enzyme Digested Chicken

Oligopeptide	Pepsin	Chymotrypsin	Trypsin	Thermolysin
LKPNM				0.4
LKPN	0.1			1.1
LKP				0.3
LK			0.7	0.3
KPNM				0.2
PNM				0.5

yield rate > 0.1 mg/g fresh food

Concentration of oligopeptides released = mg oligopeptide/g fresh food.

Table 4: Oligopeptides Released from Enzyme Digested Turkey

Oligopeptide	Pepsin	Chymotrypsin	Trypsin	Thermolysin
LKPNM				0.2
LKPN				1
LKP				0.3
LK			0.7	0.3
KPNM				0.1
PNM				0.4

yield rate > 0.1 mg/g fresh food

Concentration of oligopeptides released = mg oligopeptide/g fresh food.

Table 5: Oligopeptides Released from Enzyme Digested Fish

Oligopeptide	Pepsin	Chymotrypsin	Trypsin	Thermolysin
LKPNM	0.6	0.1	0.2	0.4
LKPN	1.1		0.1	0.8
LKP	0.3			0.2
LK	0.6	0.7	0.5	0.2
KPNM	0.3	0.1		0.3
PNM	0.7	0.1	0.1	0.4

yield rate > 0.1 mg/g fresh food

Concentration of oligopeptides released = mg oligopeptide/g fresh food.

Table 6: Oligopeptides Released from Enzyme Digested Soy

Oligopeptides	Pepsin	Chymotrypsin	Trypsin	Thermolysin
LKPNM				
LKPN				
LKP				
LK				
KPNM				
PNM				0.2

yield rate > 0.1 mg/g fresh food

Concentration of oligopeptides released = mg oligopeptide/g fresh food

Example 3: Liquid Phase Synthesis of Leucyl-Lysyl-Proline (LKP)

Step 1: Preparation of N^{α}-Fmoc-N^{ε}-Boc-Lysyl-Proline *tert*-Butyl Ester

White solid of N,N'-Disuccinimidyl carbonate (3 g, 12 mmol) was suspended in the solution of Fmoc-Lys(Boc)-OH (4.5 g, 10 mmol) in 20 ml of DMF. When 200 mg of DMAP was added to above mixture, gas evolved and the solids dissolved. The clear mixture was stirred at room temperature for 4 hours and the liquid Pro-OtBu (1.7 g, 10 mmol) was added dropwise. After stirring at room temperature overnight, the solution was evaporated to dryness under reduced pressure at 35°C. The desired product was purified by prep HPLC and lyophilized as a white powder (4.4 g, 70%).

Step 2: Preparation of Boc-Leucyl-N$^{\varepsilon}$-Boc-Lysyl-Proline *tert*-Butyl Ester

4.4 g of the product prepared in step 1 was dissolved in 50 ml of 20% piperidine in DMF. The reaction mixture was stirred for 40 minutes, and concentrated to dryness. The residue was re-dissolved in 40 ml of DMF. To this solution was added Boc-Leu-OSu (2.3 g, 7 mmol). After allowing the reaction to proceed overnight, the mixture was evaporated to dryness under reduced pressure at 35°C. The desired product was purified by prep HPLC and lyophilized as a white powder (2.04 g)

Step 3: Preparation of Leucyl-Lysyl-Proline

Boc-Leucyl-N$^{\varepsilon}$-Boc-Lysyl-Proline *tert*-Butyl Ester prepared in step 2 was de-protected by treating it with 90% TFA in water (10 ml) for 1 hour. The final desired oligopeptide was purified by prep HPLC and lyophilized to give a white powder (1.46 g).

Example 4: Synthesis of Lysyl-Prolyl-Asparaginyl-Methionine (KPNM)

KPNM (494 mg, 69% yield) was prepared using substantially the same procedure as described in Example 3. The synthesis was started with a Fmoc-Met resin (1.0 mmol) and subsequent coupling of Fmoc-Asn(Mtt), Fmoc-Pro and Fmoc-Lys(Boc).

Example 5: Synthesis of Lysyl-Prolyl-Asparagine (KPN)

KPN (434 mg, 92% yield) was prepared using substantially the same procedure as described in Example 3. The synthesis was started with a Fmoc-Asn(Trt) resin (1.0 mmol) and subsequent coupling of Fmoc-Pro and Fmoc-Lys(Boc).

Example 6: Synthesis of Leucyl-Lysine (LK)

LK was prepared using substantially the same procedure as described in Example 3. The synthesis was started with a Fmoc-Lys(Boc) resin (1.0 mmol) and coupled with Fmoc-Leu. After synthesis, the resulting peptide resin was treated with TFA (20 mL), for 1 h. The desired product (389 mg, 80% yield) was obtained as a white powder.

Example 7: Synthesis of Asparaginyl-Methionine (NM)

To a stirred solution of Boc-Asn-OH (2 mmol) and Met-OMe.HCl (0.4 g, 2 mmol) in DMF (10 mL) was added HOBt (2.2 mmol), HBTU (2 mmol) and DIEA (6 mmol). The mixture was stirred at room temperature overnight. After the solvent was removed, Boc-Asn-Met-OMe was purified by prep-HPLC. The protecting groups were removed first with a mixture of TFA/CH_2Cl_2 (V/V=95/5) and then with the solution of 0.5 N LiOH

in 12.5 mL methanol. Prep-HPLC purification and lyophilization afforded TFA•Asn-Met-OH (70 mg, 9 % overall yield) as a white solid.

Example 8: *In vitro* ACE inhibition Assay

KPNM, KPN, PNM, LK, PN and NM were synthesized as discussed above and analyzed using an *in vitro* ACE inhibition assay using a 96-well plate format. N-(3-[2-furyl]acryloyl)-Phe-Gly-Gly (FAPGG) was used as the substrate, which had an absorbance at 340 nm. The decrease in absorbance of FAPGG was followed during the reaction to determine the enzyme activity.

A mixture containing 100 μl of 1mM FAPGG, 50 μl of the oligopeptide compound (in different concentrations starting from 10 mM with a series of 1:3 dilutions repeated six times), and 25 μl of reaction buffer (50 mM HEPES, pH 7.4, 0.3 M NaCl) was added to each well and pre-warmed at 37°C. 25 μl of 0.2 U/ml rabbit lung angiotensin converting enzyme (Sigma Chemical Company, St. Louis, MO) was added to the mixture to start the reaction. Reactions were carried out at 37°C for 8 minutes and the absorbance of the mixture was read at 340 nm to determine enzyme activity. A reaction mixture containing FAPGG and reaction buffer (50 mM HEPES, pH 7.4, 0.3 M NaCl) was included in each assay and used as a control. This procedure was conducted for each oligopeptide of interest.

The concentration of oligopeptide at the inhibition rate of 50% of reaction control is referred to as the IC_{50} value.

The results of the *in vitro* ACE inhibition assay are set out in Table 7 below. As can be seen, LKPNM, LKP and KP demonstrated ACE inhibition *in vitro*.

Table 7: ACE Inhibition *in vitro*

Oligopeptide	IC_{50} (μM)
LKPNM	10
LKPN	2237
KPNM	1035
LKP	4
KPN	>3500
PNM	>3500
LK	>3500
KP	51
PN	>3500
NM	>3500

Example 9: Intravenous Administration of Oligopeptides *In Vivo*

The effects of KPNM, KPN, PNM, LK, PN and NM on systolic blood pressure was also evaluated *in vivo*. KPNM, KPN, PNM, LK, PN and NM were synthesized as described above, dissolved in physiological saline and aseptically filtered through a filter. Catheters were placed in the femoral veins of the animals for intravenous infusion. The resulting filtrate containing the desired oligopeptide was intravenously administered to the animals. Ten μmoles of KPNM, KPN, PNM, LK, PN and NM were intravenously administered to spontaneously hypertensive rats (Taconic, Germantown, New York, about 350g each, 6 animals per group) and the change in blood pressure immediately following i.v. administration was compared with that of a control group to which physiological saline was intravenously administered. The change in blood pressure was also compared to that of a control group to which Captoril was intravenously administered. The doses were delivered intravenously in about 1 ml volume over a five minute period. Blood pressure was monitored continuously throughout the experiment through a catheter placed in the femoral artery using a polygraph. Graphic results of this study can be seen in Figure 1.

This same procedure was repeated using an intravenous dosage of five μmoles of KPNM, KPN, PNM, LK, PN and NM. The graphic results of this study can be seen in Figure 2.

These experiments demonstrate that an intravenous dose of the oligopeptides will reduce blood pressure in the SHR model. The results of these tests for the oligopeptides of this invention are shown in Table 8.

Table 8: Effect of Intravenous Administration of Synthesized Oligopeptides in SHR Rats

Compound	Mean SBP Lowering (mmHg) 10µmol/ml/rat	Mean SBP Lowering (mmHg) 5µmol/ml/rat
Saline	1.63	0.00
Captoril	52.67	39.60
KPNM	14.89	30.50
PNM	17.00	35.50
KPN	18.38	39.50
LK	28.63	25.30
PN	4.63	1.50
NM	6.88	21.50

Example 10: Effect of Oral Administration of Oligopeptides on Systolic Blood Pressure

27 week old male spontaneous hypertensive rats (SHR) (Taconic, Germantown, New York, about 500g each, 6 animals per group) were used to further test the antihypertensive properties of these oligopeptides *in vivo*. KPNM, KPN, PNM, LK, PN and NM were synthesized as described above in the Examples above, dissolved in distilled water and aseptically filtered through a filter to obtain a filtrate containing the desired oligopeptide to be administrated orally. 10 µmoles/rat of each of the oligopeptides was orally administered to each animal of group. 1 ml of distilled water were orally administered to animals of one control group and 10 µmoles/rat of Enalapril was orally administered to animals of a second control group. Blood pressure was measured before the administration of the compounds and after 4 hours by a tail cuff method using a noninvasive blood pressure apparatus (Twelve channel Semi-Automatic NIBP Test System, IITC Inc.). The change in the maximal blood pressure in the animals of each group treated with the oligopeptides was compared with the blood pressure of animals of the control groups to which distilled water and Enalapril was orally administered. Graphic results of this study are shown in Figure 3.

This same procedure was repeated using 13 week old SHR model. Graphic results of this study are shown in Figure 4.

In light of the detailed description of the invention and the examples presented above, it can be appreciated that the several aspects of the invention are achieved.

It is to be understood that the present invention has been described in detail by way of illustration and example in order to acquaint others skilled in the art with the invention, its principles, and its practical application. Particular formulations and processes of the present invention are not limited to the descriptions of the specific embodiments presented, but rather the descriptions and examples should be viewed in terms of the claims that follow and their equivalents. While some of the examples and descriptions above include some conclusions about the way the invention may function, the inventors do not intend to be bound by those conclusions and functions, but put them forth only as possible explanations.

It is to be further understood that the specific embodiments of the present invention as set forth are not intended as being exhaustive or limiting of the invention, and that many alternatives, modifications, and variations will be apparent to those skilled in the art in light of the foregoing examples and detailed description. Accordingly, this invention is intended to embrace all such alternatives, modifications, and variations that fall within the spirit and scope of the following claims.

We claim:

1. An anti-hypertensive peptide in substantially pure form selected from the group of peptides having the amino acid sequence Lys-Pro-Asn-Met, Lys-Pro-Asn, Pro-Asn-Met, Leu-Lys, Pro-Asn and Asn-Met.

2. An anti-hypertensive composition which comprises at least one member which is selected from the group consisting of Lys-Pro-Asn-Met, Lys-Pro-Asn, Pro-Asn-Met, Leu-Lys, Pro-Asn and Asn-Met or its pharmacologically acceptable acid or base addition salt and is in an amount effective to reduce hypertension in a subject.

3. A pharmaceutical composition comprising at least one member selected from the group consisting of Lys-Pro-Asn-Met, Lys-Pro-Asn, Pro-Asn-Met, Leu-Lys, Pro-Asn and Asn-Met or its pharmacologically acceptable acid or base addition salt and a pharmaceutically acceptable carrier.

4. An edible composition comprising a food stuff comprising a composition selected from the group of oligopeptides consisting of Lys-Pro-Asn-Met, Lys-Pro-Asn, Pro-Asn-Met, Leu-Lys, Pro-Asn and Asn-Met or its pharmacologically acceptable acid or base addition salt.

5. The edible composition of claim 4 wherein the composition is an antihypertensive composition.

6. The edible composition of claim 5 wherein the antihypertensive composition is in an amount effective to reduce hypertension in a mammal.

7. The edible composition of claim 6 wherein the mammal is human.

8. The edible composition of claim 4 wherein the foodstuff comprises beverages, infused foods, sauces, condiments, salad dressings, fruit juices, syrups, desserts, icings and fillings, soft frozen products, confections, chewing gum and intermediate food.

9. The edible composition of claim 4 wherein the edible composition further comprises a nutritional substance.

10. The edible composition of claim 4 wherein the edible composition is substantially a liquid.

11. The edible composition of claim 4 wherein the edible composition is substantially a solid.

12. The edible composition of claim 4 wherein the edible composition is a gelatin.

13. The edible composition of claim 4 wherein the edible composition is a milk-based composition.

14. A method for treatment or prophylaxis of hypertension in a subject, said method comprising administering to the subject an antihypertensively effective amount of the composition of claim 3.

15. A method for treatment or prophylaxis of myocardial infarction caused by or associated with hypertension in a subject, said method comprising administering to the subject an antihypertensively effective amount of at least one member selected from the group consisting of Lys-Pro-Asn-Met, Lys-Pro-Asn, Pro-Asn-Met, Leu-Lys, Pro-Asn and Asn-Met or its pharmacologically acceptable acid or base addition salt.

16. A method for treatment or prophylaxis of left ventricular systolic dysfunction caused by or associated with hypertension in a subject, said method comprising administering to the subject an antihypertensively effective amount of at least one member selected from the group consisting of Lys-Pro-Asn-Met, Lys-Pro-Asn, Pro-Asn-Met, Leu-Lys, Pro-Asn and Asn-Met or its pharmacologically acceptable acid or base addition salt.

17. A method for treatment or prophylaxis of diabetes mellitus caused by or associated with hypertension in a subject, said method comprising administering to the subject an antihypertensively effective amount of at least one member selected from the group consisting of Lys-Pro-Asn-Met, Lys-Pro-Asn, Pro-Asn-Met, Leu-Lys, Pro-Asn and Asn-Met or its pharmacologically acceptable acid or base addition salt.

18. A method for treatment or prophylaxis of progressive renal failure caused by or associated with hypertension in a subject, said method comprising administering to the subject an antihypertensively effective amount of at least one member selected from the group consisting of Lys-Pro-Asn-Met, Lys-Pro-Asn, Pro-Asn-Met, Leu-Lys, Pro-Asn and Asn-Met or its pharmacologically acceptable acid or base addition salt.

19. A method for treatment or prophylaxis of congestive heart failure caused by or associated with hypertension in a subject, said method comprising administering to the subject an antihypertensively effective amount of at least one member selected from the group consisting of Lys-Pro-Asn-Met, Lys-Pro-Asn, Pro-Asn-Met, Leu-Lys, Pro-Asn and Asn-Met or its pharmacologically acceptable acid or base addition salt.

FIG. 1

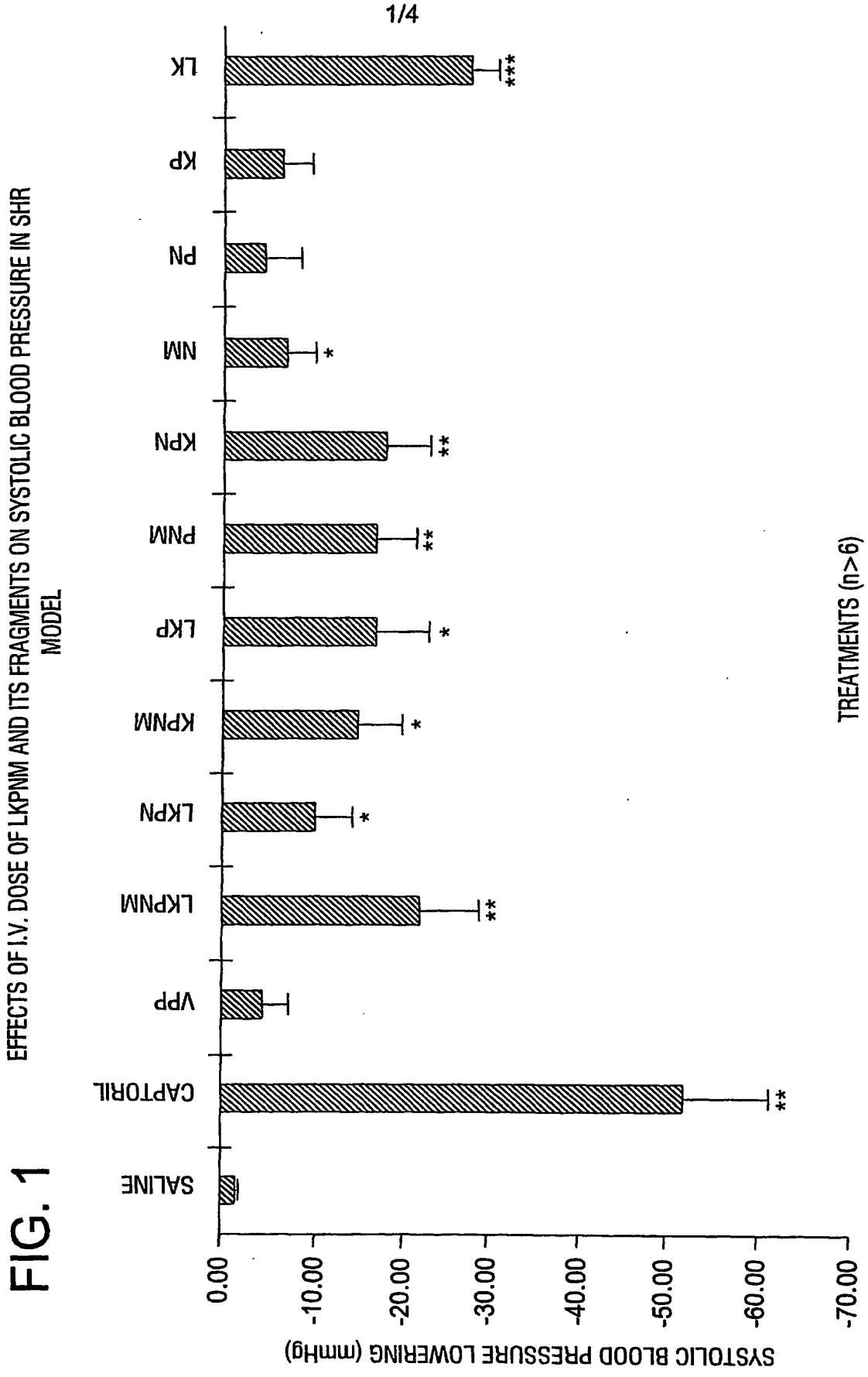
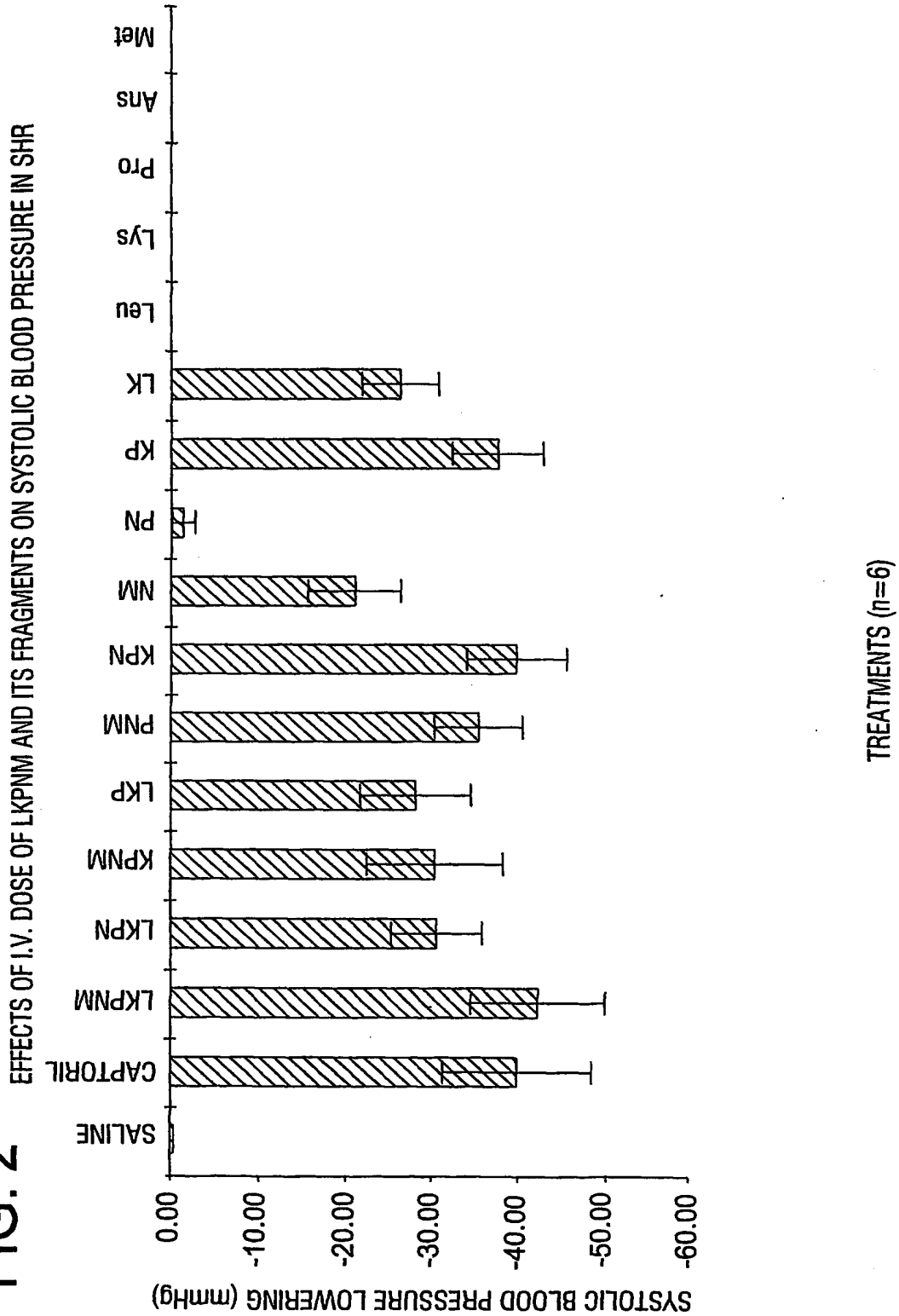


FIG. 2



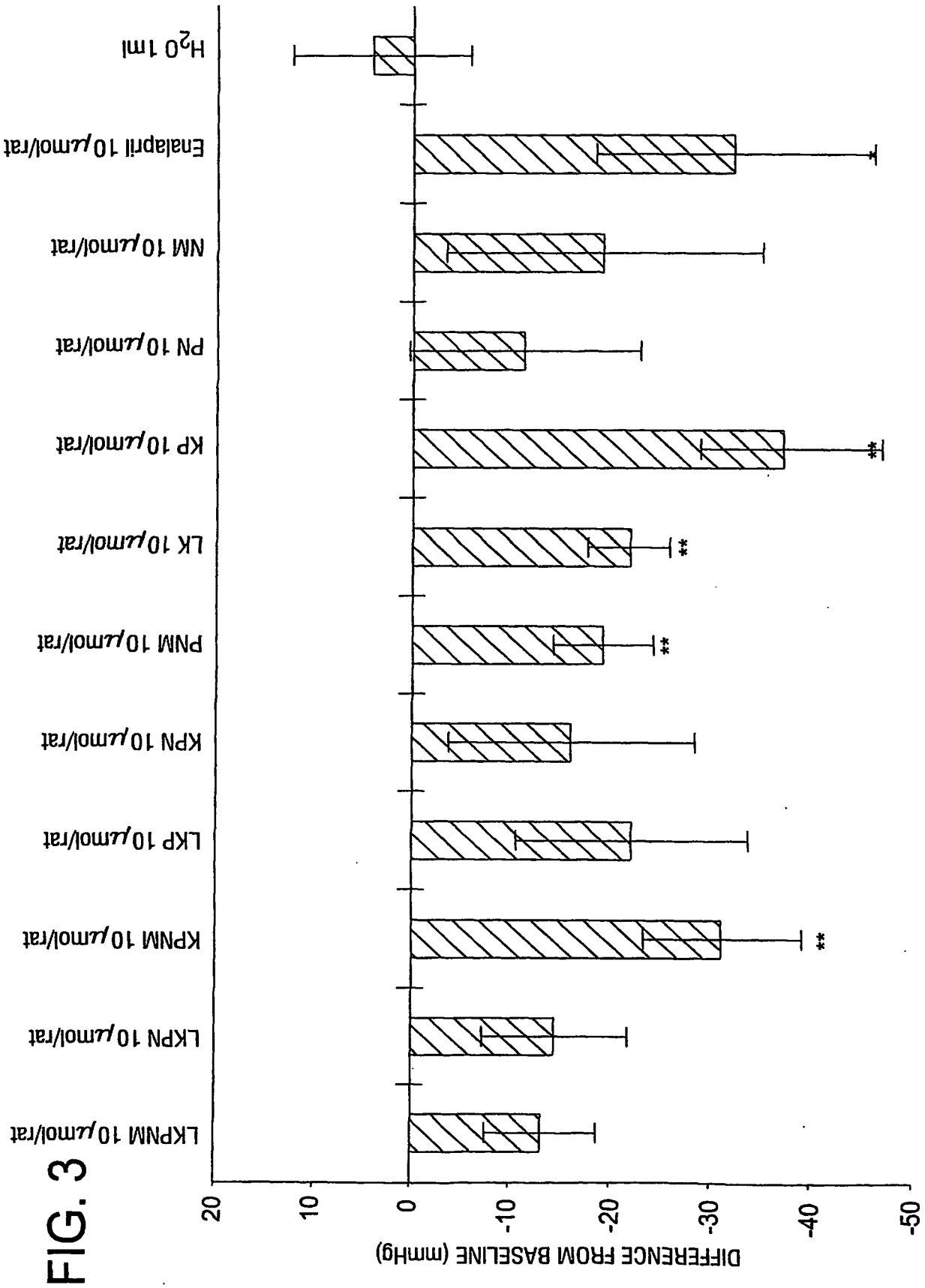


FIG. 3

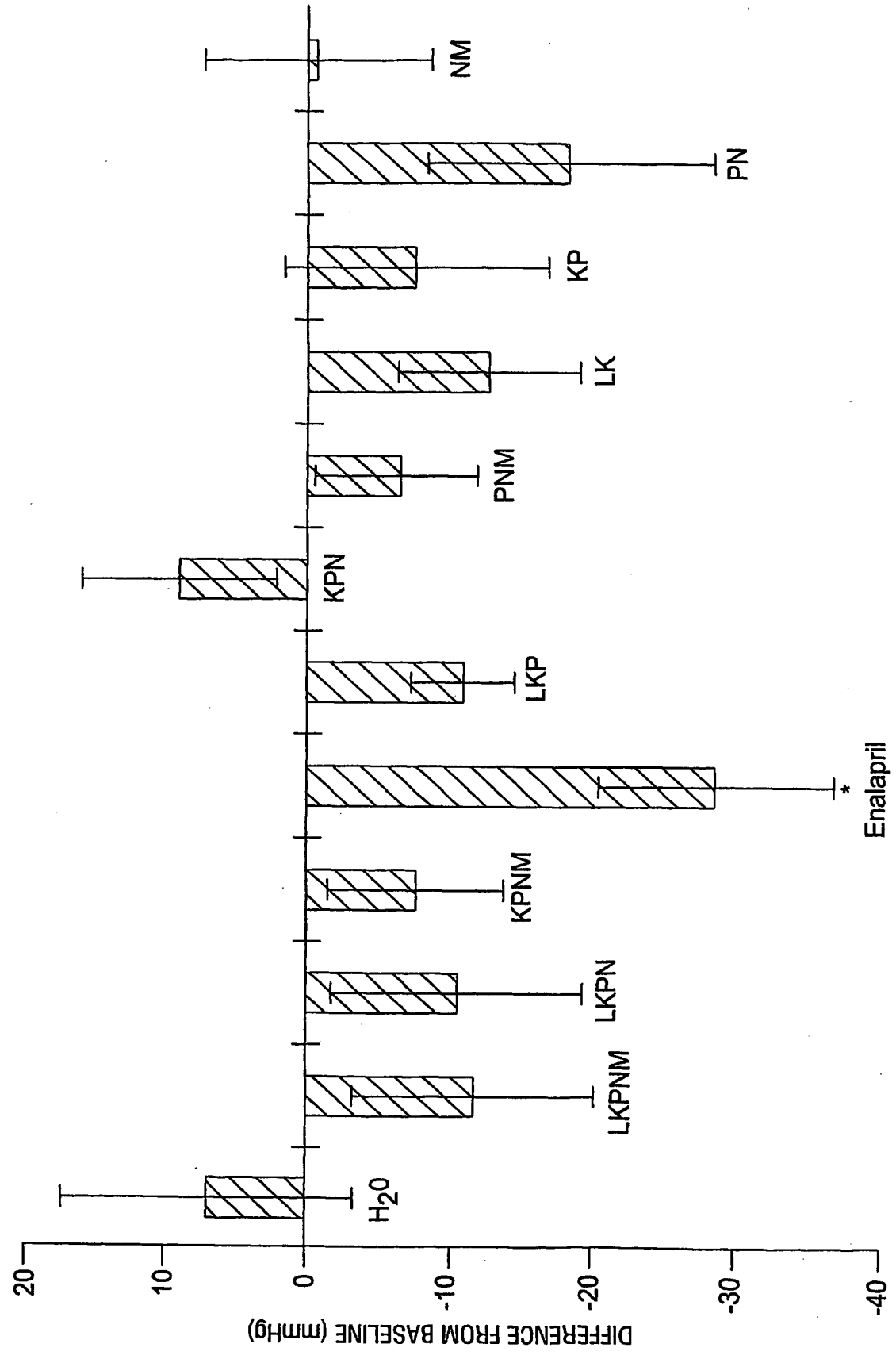


FIG. 4

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<110> Guan, Zhonghong
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Mandrell, Kathy
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<120> ANTI-HYPERTENSIVE PEPTIDES

<130> MTC 6617.1

<150> 60/188,499
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/07479

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 38/00, 38/06; C07K 5/00, 5/10; A23C 9/12
US CL : 514/2, 18; 530/331, 344, 354; 426/34, 580, 590, 656

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/2, 18; 530/331, 344, 354; 426/34, 580, 590, 656

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 04-069397 A2 (NIPPON GOSEI KAGEKU KOGYO K.K.) 04 February 1992 (04.03.92) , see attached English abstract.	1-6, 8-11, 14
X	JP 08-225593 A2 (SUETSUNA YOKO) 03 September 1996 (03.09.96), see attached English abstract.	1-5, 8,9,11,14
X	JP 2000060489 A2 (NIPPON SYNTHETIC CHEMICAL INDUSTRY CO. LTD.) 29 February 2000 (29.02.00), see attached English abstract	1-6, 8-11, 14
X,P	SAITO, T. et al. Isolation and Structural Analysis of Antihypertensive Peptides That Exist Naturally in Gouda Cheese. J. Dairy Science, July 2000, Vol. 83, No. 3, pages 1434-1440. See abstract, pages 1436-1439, Fig. 1 and 2, Tables 2 and 3.	1-6, 8,9,11,13,14
Y	NAKAMURA et al. Antihypertensive Effect of Sour Milk and Peptides Isolated from It That Are Inhibitors to Angiotensin I-Converting Enzyme. J. Diary Sci. 1995, Vol. 78, No. 6, pages 1253-1257. See abstract, pages 1254-1255, Fig. 1 and 2.	3-6, 8-11, 13-15
Y	US 5,854,029 A (YAMAMOTO) 29 December 1998. See columns 2-4, Examples 1-3.	3-6, 8-11,13-15
A	US 5,691,310 A (VESELY) 25 November 1997. See columns 3-6, Fig 2-5, Examples.	14,15,18,19

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search
03 August 2001 (03.08.2001)

Date of mailing of the international search report
27 AUG 2001

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Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/07479

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	CASARINI et al. Angiotensin Converting Enzymes from Human Urine of Mild Hypertensive Untreated Patients Resemble the N-Terminal Fragment of Human Angiotensin I-Converting Enzyme. Int. J. Biochem. Cell Biology. January 2001, Vol. 33, pages 75-85. See the whole document.	1-19

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/07479

Continuation of B. FIELDS SEARCHED Item 3:
STN search on CAPLUS, MEDLINE, EMBASE, BIOSIS and SCISEARCH; EAST search on USPAT, DERWENT, EPO, JPO. Search term used: antihypertensive, hypertensive, hypertension, polypeptide, peptide, treating, treatment, prophylaxis, composition, food, ingestible, myocardial infarction, human, mammal, ventricular systolic dysfunction, renal failure, renal impairment, diabetes mellitus, congestive heart failure. Amino acid sequence search for Lys-Pro-Asn-Met, Lys-Pro-Asn, Pro-Asn-Met, Leu-Lys, Pro-Asn and ASn-Met.